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Damage avoidance and repair mechanisms of extreme halophiles to ionizing radiation

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AFOSR Final Report

Title: Damage avoidance and repair mechanisms of extreme halophiles to ionizing radiation

Grant/Contract Number: FA9550-10-1-0057

Reporting period: April 2010- April 2013

PI: Jocelyne DiRuggiero – Johns Hopkins University

Abstract

We investigated the molecular basis for ionizing radiation (IR) resistance in extremophiles using the halophilic archaeon *Halobacterium salinarum* as a model system and several thermophilic bacteria and archaea also highly resistant to IR. Oxidative damages are the main lesions from IR and result from the production of reactive oxygen species (ROS) via radiolysis of water.

Our work with halophiles showed that in *H. salinarum* IR resistance is achieved by non-enzymatic processes, effected by manganese (Mn) antioxidants and coordinated by regulatory networks. While ROS-scavenging enzymes were essential for resistance to chemical oxidants, these enzymes were not necessary for *H. salinarum*'s resistance to IR. Enzyme-free cell extracts of *H. salinarum* protected protein activity up to 10 kGy of IR and contained high levels of Mn, phosphates, and amino acids, supporting an essential role in ROS scavenging for those small molecules *in vivo*. Biochemical analysis of "super-IR resistant" (IR^+) mutants of *H salinarum* and *Haloferax volcanii*, evolved over multiple cycles of exposure to high doses of IR, confirmed the key role for Mn antioxidants in the IR resistance of these organisms. Analysis of the proteome of *H. salinarum* IR^+ mutants revealed increased expression for proteins involved in central metabolism and energy generation, channeling a substantial flux of carbon into pyruvate and therefore the generation of energy and reducing equivalents. Maintenance of redox homeostasis was also activated by the over-expression of coenzyme biosynthesis pathways involved in redox reactions. Increased levels of expression of proteins involved in DNA metabolism also indicated a significant role for RPA proteins in the enhanced repair of DNA strand breaks resulting from IR exposure.

The diversity of IR resistance mechanisms was investigated by analyzing the properties of the enzyme-free cell extracts of IR resistant thermophilic bacteria and anaerobic hyperthermophilic archaea. Our data showed that high Mn concentrations combined with the presence of trehalose in the intracellular milieu of the *Rubrobacter* species provide these organisms with antioxidants that are key for the high IR resistance we observed. In contrast, *P. furiosus* and *T. gammatolerans* have low intracellular concentrations of Mn and we found little ROS scavenging activity from DIP and MG at physiological conditions. We propose that their IR resistance results from their adaptation to an anaerobic life style with low IR-induced ROS production because of the absence of oxygen, and efficient O_2 detoxification enzymes that are required for their survival.

This work established that IR resistance in extremophiles is the result of the coordination of multiple, tightly regulated metabolic pathways that are linked to the adaptation of organisms to their environment.

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1. PROGRESS REPORT

Oxidative stress occurs when the level of reactive oxygen species (ROS) produced in cells by aerobic metabolic activity or environmental challenges overwhelm antioxidant defense mechanisms and damage accumulates. In this context, ionizing radiation (IR) is of particular interest because most of its deleterious effects result from the production of ROS via radiolysis of water, imparting severe oxidative stress to all the cell's components.

Radiation resistant bacteria have garnered a great deal of attention from scientists seeking to expose the mechanisms underlying their incredible survival abilities. Early work on the desiccation resistance of extremely radiation-resistant bacteria revealed that it is the adaptation to extremely dry environments and high tolerance to desiccation that imparts IR resistance to these organisms. The distribution of extremely IR and desiccation resistant organisms in the phylogenetic tree of life is not limited to bacteria and a number of studies have revealed high levels of resistance for several archaea and eukarya.

Critical work by us (Robinson et al., 2011) and others (Daly et al., 2010) have promoted the model of protein protection from ROS as the main factor in IR resistance, as opposed to classical models of efficient DNA repair. Key to this model is the concept that IR resistant organisms accumulate in their cytoplasm small molecules such as manganese complexes, orthophosphate, nucleosides/bases, amino acids (aa), and peptides that are able to scavenge ROS and prevent extensive damage to proteins. Functional DNA repair enzymes can then repair DNA double-strand breaks (DSBs) and oxidized nucleotides resulting from IR before cell death occurs. We showed that the halophilic archaeon, *Halobacterium salinarum,* is highly resistant to IR and that it accumulates Mn^{2+}, aa, and peptides at much higher levels than IR sensitive organisms (Robinson et al., 2011).

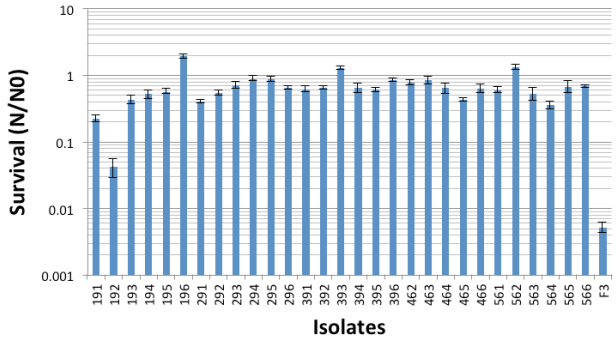


Fig. 1. Survival of *H. salinarum* IR^+ isolates at 12 kGy of IR. Survival was expressed as the average ratio (N/N0) of cfu/ml from irradiated (N) compared to un-irradiated (N0) cultures. Data are the averages of three independent experiments with triplicate measurements each; standard errors shown. Founder F3 was added for comparison.

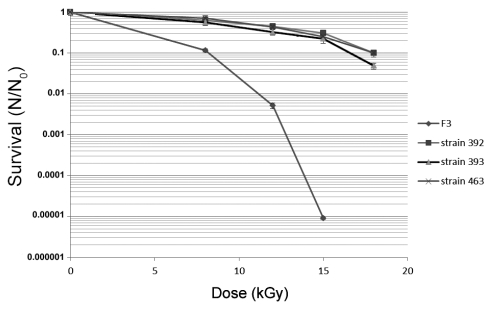


Fig. 2. Survival of IR^+ isolates and founder strain F3 at increasing doses of IR. Survival is expressed as in Fig. 1. Data are the averages of three independent experiments with triplicate measurements each; standard errors shown.

The work below elucidate further the molecular mechanisms and metabolic pathways involved in the IR resistance in halophilic archaea. We addressed the question of diversity of IR resistance mechanisms by investigating IR resistant thermophilic bacteria and anaerobic hyperthermophilic archaea.

1.1 Evolution and characterization of IR$^+$ isolates of *H. salinarum* (Robinson et al., 2011; Webb et al., 2013)

We selected for super-IR resistant (IR$^+$) isolates of *H. salinarum* to further investigate the mechanisms contributing to the radiation resistance of this halophile. Five individual cultures (founders F1 to F5) were sequentially irradiated with high levels of gamma-radiation. After completion of the irradiation-recovery rounds, a survival test using 12 kGy as a benchmark showed 60% survival for 3 of the evolved cultures, and 42% and 23% survival for 2 other evolved cultures, respectively.

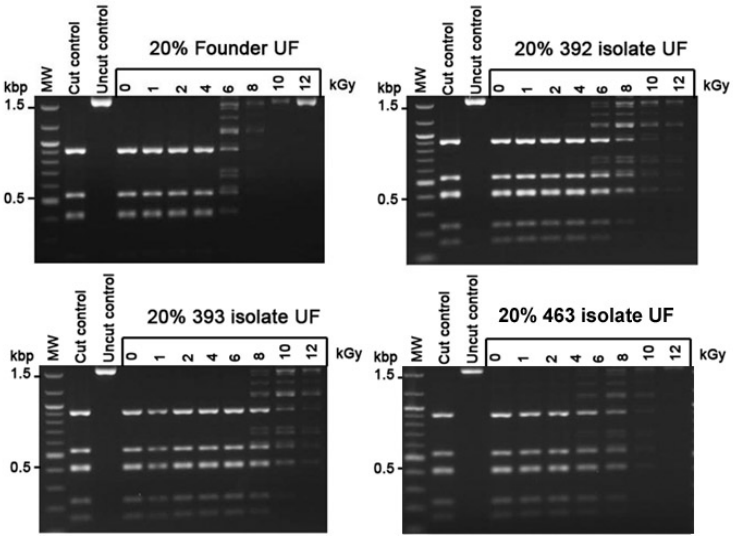


Fig. 3. Protection of enzyme activity. The restriction enzyme *Dde*I was irradiated up to 12 kGy in UFs of *H. salinarum* IR$^+$ isolates and founder strain F3. Residual restriction enzyme activity was assayed by the digestion of pUC19 plasmid DNA. Fragments were analyzed by agarose gel electrophoresis. The first lanes are molecular size ladders.

Thirty isolates from these mixed cultures were randomly selected and tested for their survival at 12 kGy; survival for 23 of the 30 isolates was higher than 50% and all had significant increases in IR resistance when compared to the founder (F3) strain (Fig. 1). Isolates 392, 393, 463 were selected for further investigation; Founder 3 (F3) was used for comparison. The D_{10} - dose at which 10% of cells survive - was calculated from survival curves for each of the 3 isolates and revealed an increase from 5 to 17 kGy when compared with F3 (Fig. 2). This was a significant increase in IR resistance and these strains were used for biochemical characterization,

1.2 Biochemical analysis of *H. salinarum* IR$^+$ isolates (Webb et al., 2013)

To determine the role of antioxidant molecules in the increased IR resistance of *H. salinarum* IR$^+$ isolates, we tested the ability of enzyme-free cell extracts (ultrafiltrates, UFs) from isolates 392, 393 and 463, and from F3, to protect enzyme activity from IR, *in vitro*. The restriction enzyme *Dde*I was irradiated at increasing doses of IR, in presence of the UFs, and its residual activity determined by restriction of plasmid DNA and

analysis of the fragments by gel electrophoresis. Residual enzyme activity was detected up to 6 kGy with UF_{F3} and up to 10 to 12 kGy with UFs from the 3 IR^+ isolates, demonstrating significant increased in IR protection *in vitro* (Fig. 3). While we observed a loss of cleavage site specificity for the enzyme at the highest doses, the doses at which this, and the total loss of protein activity, occurred were consistent across the IR^+ isolates and F3.

Accumulation of intracellular manganous (Mn^{2+}) ions forming antioxidant complexes with peptides, orthophosphate, and other small molecules, and catalytically scavenging ROS, was previously demonstrated for *H. salinarum* and *D. radiodurans* (Daly et al. 2010; Robinson et al. 2011). We measured the concentrations of Mn, PO_4, and aa in our 3 IR^+ isolates (Fig. 4). Isolates 392 and 463 had 1.5-fold and 1.3-fold increased Mn in their UFs, respectively, when compared to UF_{F3} ($p < 0.05$; Fig. 4a), and UF_{392} had 1.5-fold more PO_4 and 2.5-fold more amino acids than UF_{F3}, ($p < 0.05$; Fig. 4b and c). Compositional analysis of amino acids showed that the concentration of glycine in UF_{392} was 3 times that UF_{F3} (Fig. 4d). The UF from isolate 393 had concentration of Mn, PO_4, and amino acids comparable to that of F3 (Fig. 4).

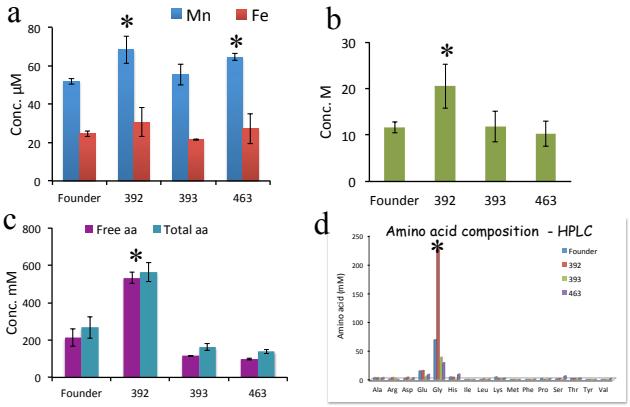


Fig. 4. Mn and Fe (a), phosphate (b), total and free amino acids (c), and amino acid composition (d) measured in the enzyme-free UFs of *H. salinarum* IR^+ isolates and founder strain F3. Data for (a) to (c) are the averages of at least three experimental replicates with triplicate measurements each; standard errors shown. * $P < 0.01$ (Student's *t* test).

Amino acids and small peptides have been shown to have ROS scavenging capabilities *in vitro* and *in vivo* and it suggests that, in isolate 392, the combination of high Mn, PO_4, and glycine might be an important component of the enhanced IR resistance of this strain. While isolate 463 also displayed increased intracellular Mn, this was not the case for isolate 393, indicating that other organic metabolites with radioprotective properties might be found in these cells. There are indeed several examples of metabolites with ROS scavenging properties among the IR-resistant organisms investigated so far, including aa, nucleosides and small peptides (Daly et al. 2010; Robinson et al. 2011), trehalose, dipicolinic acid, and mycosporin-like amino acids. However, any biochemical analyses remain challenging in *H. salinarum* because of the extremely high KCl concentration typically found in these cells.

1.3 Proteomic analysis of *H. salinarum* IR^+ isolates (Webb et al., 2013)

We used a proteomic approach to identify proteins and metabolic pathways that contributed to the enhanced IR resistance observed in the IR^+ isolates. Differentially expressed proteins in the IR^+ isolates versus the founder strain were identified using the

iTRAQ method. We selected a proteomic approach to circumvent temporal shifts between transcripts and protein abundance levels previously reported for *H. salinarum* in response to IR (Whitehead et al., 2006). The analysis produced 386,843 spectra. Using the GenBank database we identified 126,845 peptides that formed 1,266 protein groups and 1,279 merged proteins, covering 48% of the *H. salinarum* proteome.

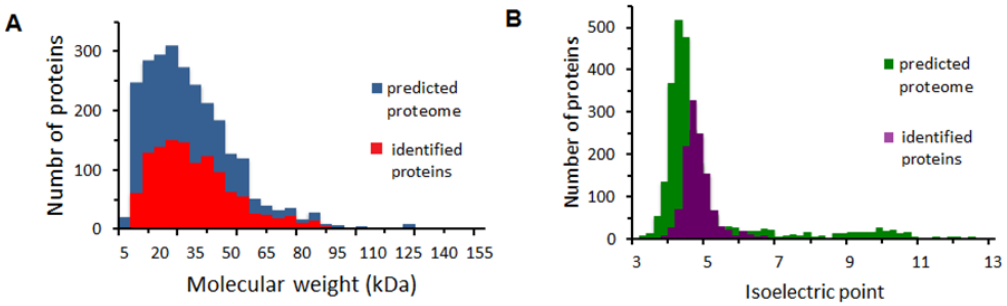


Fig. 5. iTRAQ proteome analysis. (A) Distribution of protein molecular weights in the predicted proteome of *H. salinarum* and the iTRAQ dataset of identified proteins. (B) Distribution of isoelectric points of the predicted proteome of *H. salinarum* and the iTRAQ dataset of identified proteins.

Physical properties such as isoelectric point and molecular weight of the proteins identified in the iTRAQ dataset were compared with that of the predicted proteome of *H. salinarum* (Fig. 5). Both the molecular weights and the pI values of our iTraq-identified proteins indicated that our dataset was a good representation of the *H. salinarum* proteome without notable biases.

The iTRAQ dataset of identified proteins and predicted proteome of *H. salinarum* were sorted into 18 metabolic categories (CMR database) (Fig. 6). Hypothetical and unclassified proteins were the largest categories in both the identified and predicted proteins. Of the categories with actual metabolic function, energy metabolism had the most members in both the iTRAQ dataset of identified proteins and the predicted proteome. Over all the categories, the iTRAQ data correlated well with the predicted proteome, except for a slight underrepresentation of cell envelope proteins in the iTRAQ dataset (Fig. 6).

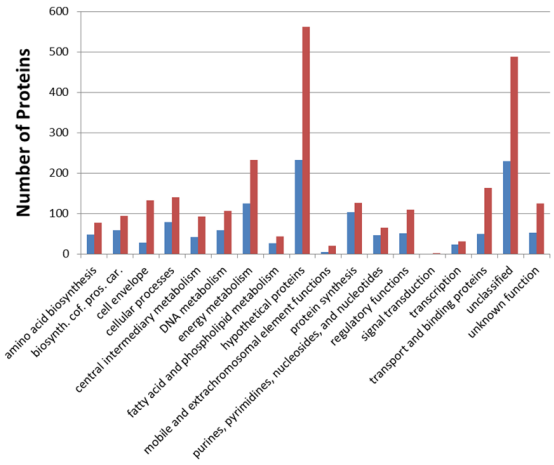


Fig. 6. iTRAQ dataset of identified proteins and predicted proteome of *H. salinarum* sorted by metabolic categories according to the CMR database. Blue, iTRAQ identified proteins; red, predicted proteome.

Using Proteome Discoverer, protein expression ratios (PER) were calculated for each protein using unique MS-identified peptides for each protein and an overall assigned score. We then identified proteins differentially expressed in the IR^{+} isolates with respect to the founder by selecting cut-off ratios of <0.4 and >1.5 for a false discovery

rate (FDR) of <6%. FDR were calculated by computing outliers in all biological replicate comparisons at specific cut-off ratios. Protein expression ratios (PER) between the four possible permutations for each isolate (isolate-1 with F3-1; isolate-2 with F3-1; isolate-1 with F3-2; and isolate-2 with F3-2) were averaged and PER <0.4 were deemed as decreased protein expression whereas PER >1.5 were deemed as increased protein expression with respect to F3.

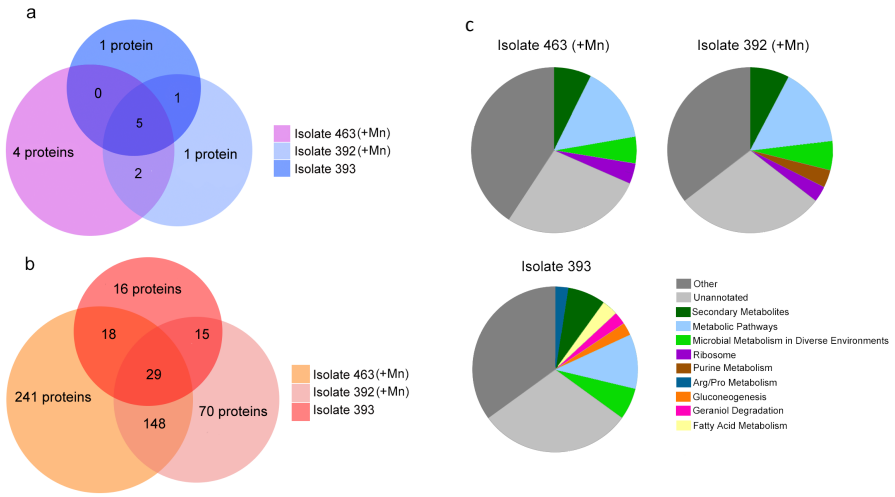


Fig. 7. Proteins with differential expression ratios (PER) identified in IR$^+$ isolates by the iTRAQ proteomic analysis. (a) Number of proteins with decreased PER. (b) Number of proteins with increased PER. (c) KEGG pathways associated with proteins with increased PER. Isolates 392 and 463 had elevated Mn concentration in their UFs when compared to the founder strain F3.

We found a total of 14 proteins with decreased PER and they fell into 4 functional categories: transcription, regulation, cellular processes, and unknown (Fig. 7). Five of the 14 proteins were common to the 3 IR$^+$ isolates, including 3 gas vesicle proteins GvpC, GvpN and GvpO, a chromosome partitioning protein SojB, and a putative signal-transducing histidine kinase/ response regulator protein HtlD. The decreased PER for gas vesicle proteins in the IR$^+$ isolates indicated a deficiency in gas vesicles biogenesis, confirmed by the sinking phenotype observed for the 3 isolates, and provided an experimental validation of our iTraq dataset. Because the three IR$^+$ isolates we characterized were gas vesicle-deficient, we tested whether the loss of gas vesicles contributed to the enhanced IR$^+$ resistance of these strains. A spontaneous

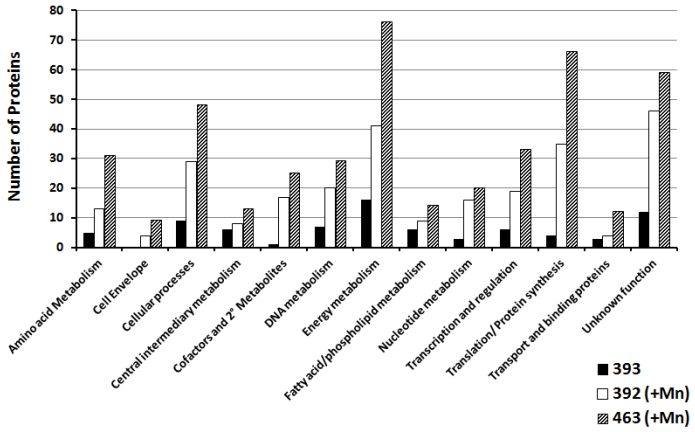
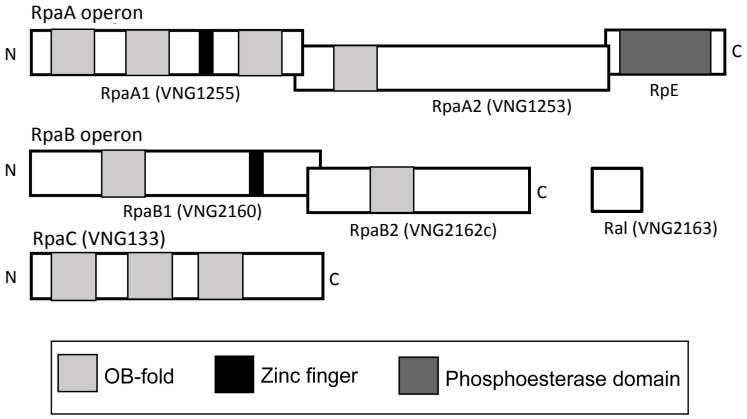


Fig. 8. Classification of protein metabolic functions for over-expressed proteins in IR$^+$ isolates. Isolates 392 and 463 had elevated Mn concentration in their UFs when compared to the founder strain F3.

F3*vac*$^-$ mutant was isolated and it radiation resistance compared to that of F3. We found no significant difference in IR resistance between the F3*vac*$^-$ mutant and F3, suggesting that the loss of gas vesicles did not contribute to increased IR$^+$ resistance in these strains.

1.4 Altered metabolic pathways in *H. salinarum* IR$^+$ isolates (Webb et al., 2013)

Five hundred and thirty seven proteins had increased PER and 29 of those were common to all IR$^+$ isolates (Fig. 7b). Among others were 2 single-strand DNA binding proteins (Rfa3, Rfa8). Isolates 392 and 463 shared the same trend with the highest number of over-expressed proteins associated with energy metabolism, cellular processes, cofactor biosynthesis, and translation/protein synthesis. "Cellular processes" was also a dominant category for common over-expressed proteins between isolates 392 and 463 (Fig. 8). One of 2 transport proteins with increased PER common to isolates 392 and 463 was a hypothetical K^+ transport system that could potential bind Mn. This is notable because both isolates 392 and 463 had increased intracellular Mn, with respect to F3, but not isolate 393 (Fig. 4a).



In parenthesis, Hbt genes; also VNG6403 and VNG5194 on minichromosomes; SSB type

Fig. 9. *H, volcanii* and *H. salinarum* gene organization for Rpa proteins. Modified from Stroud et al., 2012; Skowyra and MacNeill, 2011; Hbt gene numbers added when appropriate.

The correlation between high intracellular Mn and overall metabolic trends of over-expressed proteins between isolates 392 and 463 was investigated by classifying over-expressed proteins according to the KEGG (Kyoto Encyclopedia of Genes and Genomes) metabolic pathways Fig. 7c). Similar over-expressed metabolic pathways were found for isolates 393 and 463. These isolates accumulated significantly more Mn in their UFs than the founder strain, suggesting that the effect of intracellular Mn on cellular processes and energy metabolism might contribute to the enhanced IR resistance observed in these isolates. While the accumulation of antioxidant Mn-complexes can confer radioprotection to the cell, Mn can also enhance oxidative stress resistance by substituting as a cofactor for iron in certain enzymes susceptible to oxidative attack.

Additionally, a number of studies in bacteria reported stimulation of carbon metabolism by elevated intracellular Mn, implying that the activation of enzymes involved in energy metabolism we observed in our IR$^+$ mutants might be mediated by high cellular Mn. Additionally, recent work with the yeast *S. cerevisiae* presented evidence that intracellular Mn was tightly regulated through nutrient sensing pathways and that it was an integral part of a system level oxidative stress defense in this organism.

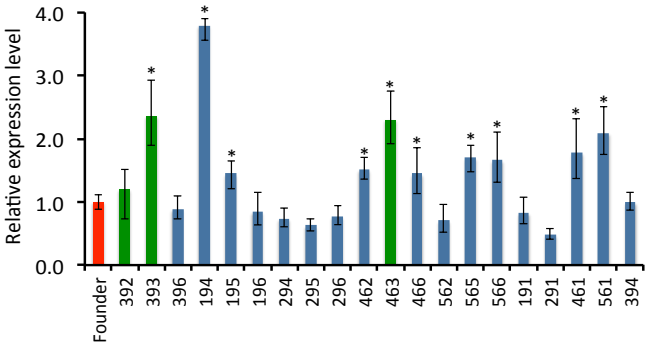


Fig. 10. mRNA expression level for *rpaC* in *Halobacterium* IR$^+$ isolates. * P < 0.01 (Student's *t* test).

Our data clearly shows activation of enzymes involved in central carbon metabolism with the over-expression of enzymes channeling a substantial flux of carbon into pyruvate and therefore the generation of energy and reducing equivalents. Maintenance of redox homeostasis was also activated by the over-expression of coenzyme biosynthesis pathways involved in redox reactions.

1.5 Overexpression of RPA proteins in *H. salinarum* (Webb et al., 2013)

While the current model regarding IR resistance is based on the cell's ability to protect its proteins from IR-induced oxidation, it is remarkable that two proteins over-expressed in all the IR$^+$ isolates were the replication factor A proteins (RPAs) Rfa3 (RpaB1 homolog) and Rfa8 (RpaB2 homolog). RPAs, also called single-strand binding proteins, bind to single stranded DNA (ssDNA) with high affinity and provide protection against nuclease and chemical attacks. RPAs are essential for DNA metabolism, including DNA replication, recombination and repair in all domains of life. Two operons, RpaA1 (RpaA homolog) and RpaA3 (RpaB homolog and including *rfa*3 and *rfa*8), and a single gene, *rfa*1 (rpaC homolog) encode RPA proteins in *H. salinarum* (Fig. 9).

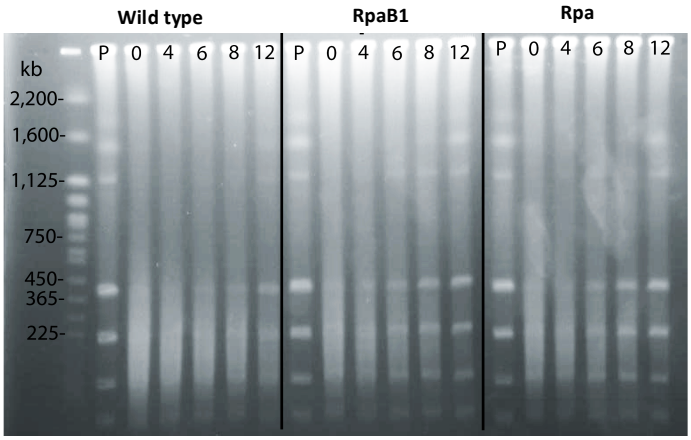


Fig. 11. PFGE time course of recovery after exposure to 2.5 kGy of radiation. Samples were taken preirradiation (P) and immediately following irradiation (0) and every 2 to 4 h during the recovery up to 12 h and were embedded in InCert agarose plugs at a final density of 10^9cells/ml. Plugs were digested with SwaI prior to gel electrophoresis

Using quantitative RT-PCR, we found over-expression of the *rpaC* gene homolog in ~50% of the *H. salinarum* IR$^+$ strains tested (Fig. 10). Increased mRNA levels for *rfa*3 and *rfa*8 were also reported for *H. salinarum* IR$^+$ mutants (DeVeaux et al. 2007). Additionally, the

*Rpa*B operon was induced in response to gamma radiation and UV-C in *H. salinarum* (Baliga et al. 2004; Whitehead et al. 2006). These data clearly implicate RPAs in enhanced survival to DNA damaging treatments.

1.6 The role of RPAs in enhanced IR resistance in halophilic archaea (Robinson et al. in preparation)

Recently, two studies reported the hypersensitivity to DNA damaging agents of deletion mutants of RpaB - but not RpaA – in *Haloferax volcanii* (Stroud et al., 2012; Skowyra and MacNeill, 2011). Constructs over-expressing the *H. volcanii* RpaC protein exhibited increased survival to UV-C, MMS, and phleomycin exposure (Skowyra and MacNeill 2012). We also showed that the strains over-expressing RpaB1 and RpaC, but not RpaA1, were more resistant to IR than the wild type (DS70) (Fig. 12 b).

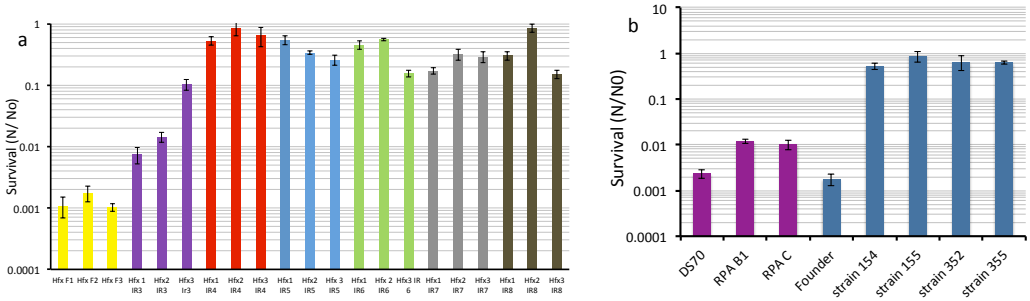


Fig. 12. Survival of *H. volcanii* strains to 2 kGy of IR. (a) IR$^+$ isolates after 1 to 8 rounds of IR at 10 kGy; Hxf1 to 3 (yellow bars) were founder strains. (b) Constructs over-expressing the RpaB1 and RpaC proteins and selected IR$^+$ isolates; DS70 is the wild type strain.

The ability to repair DNA DSBs was assayed in *H. volcanii* constructs over-expressing RpaB1 and RpaC after exposure to 2.5 kGy of IR. Samples were taken both prior to irradiation and over a time course of recovery under standard culturing conditions and analyzed by pulsed field gel electrophoresis (PFGE). In the wild type strain repair of chromosomal fragmentation after exposure to IR was initiated within 6 h and completed within 12 h (Fig. 11). In contrast, the recovery process for the RpaB1 and RpaC strains was significantly faster, with repair starting within 4 hours and completed within 8 hours (Fig. 11). It is important to note that no growth was observed for any of the strains before 8 hours of recovery. This data indicated that a major role for RPA proteins in the enhanced repair or DNA DSB resulting from IR exposure.

To further understand the role of RPAs in enhanced IR resistance, we selected for IR$^+$ isolates of *H. volcanii* using the same strategy as for *H. salinarum* (Webb and DiRuggiero, 2013). The D_{10} of the *H. volcanii* IR$^+$ isolates increased from 1.5 kGy to > 4 kGy after 4 rounds of IR at 10 kGy (Fig. 12a). We selected 30 IR$^+$ isolates after round 4 for further characterization. The IR resistance of our IR$^+$ isolates was found to be significantly higher

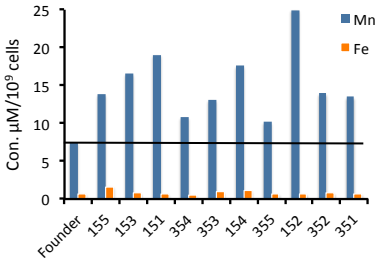


Fig. 13. Mn and Fe measured in the *H. volcanii* IR$^+$ isolates and the founder strain. Data are the averages of at least three experimental replicates.

than the *H. volcanii* constructs over-expressing the RpaB1 or RpaC proteins (Fig. 12b). We also found that all the ten *H. volcanii* IR^+ isolates tested had increased intracellular Mn when compared to the founder strain (Fig. 13). While we have not yet determine the kinetic of DNA DSB repair for the *H. volcanii* IR^+ isolates, it is likely that Mn-mediated processed are involved in the enhance IR resistance of these strains, and that over-expression of RPA proteins in itself cannot explained the level of IR resistance we observed in these mutants.

1.7 IR resistance in thermophilic bacteria and archaea (Webb and DiRuggiero, 2012)

Thermophilic bacteria and archaea inhabit diverse environments and can survive multiple stresses including desiccation, radiation, pressure and pH extremes together with high temperature. Thermophiles are distinguished by their ability to grow at or above temperatures exceeding 50°C, which demand that their macromolecules resist not only the thermal denaturing effects of heat, but also the attendant burden of elevated oxidative stress arising from metabolic processes. In this study, we investigated the role played by the compatible solutes found in two IR resistant thermophilic bacteria, *Rubrobacter xylanophilus* (D_{10} 6kGy) and *Rubrobacter radiotolerans* (D_{10} 10kGy), and two IR-resistant hyperthermophic archaea, *Pyrococcus furiosus* (D_{10} 3kGy) and *Thermococcus gammatolerans* (D_{10} 6kGy).

To determine the potential role of Mn and compatible solutes in the radiation resistance of thermophiles, we measured concentrations of metal ions, phosphates, and compatible solutes in whole cells and UFs of *R. xylanophilus, R. radiotolerans, P. furiosus,* and *T. gammatolerans* (Tables 1 and 2). UFs for the IR-resistant *Rubrobacter* species were enriched in Mn relative to that of IR-sensitive bacteria, yielding high Mn/Fe ratios similar to those found in *H. salinarum*. The concentrations of Mn found in the UFs of the anaerobic archaea *T. gammatolerans* and *P. furiosus* were more than an order of magnitude lower than the values for the *Rubrobacter* species UFs, resulting in Mn/Fe ratios similar to that of the radiation sensitive bacteria *E. coli* and *P. putida* (Table 1). The Mn/Fe ratios in whole cells followed the trend observed with the analysis of Mn/Fe ratios in the UFs (Table 1). Phosphate levels were high in all UFs with the exception of *P. furiosus* (Table 2).

Table 1. Concentrations of Mn and Fe in ultrafiltrates (UFs) and whole cells of thermophiles and radiation sensitive bacteria

			Conc. In:					
			Ultrafiltrates			Whole cells		
Organism	D_{10}[a] (kG)	Genome (Mbp)	Mn (μM)	Fe (μM)	Mn/ Fe	Mn ($ng/10^9$ cells)	Fe ($ng/10^9$ cells)	Mn/Fe
P. putida[b]	0.1	6.2	0.9	6.1	0.1	18	1045	0.02
E. coli[b]	0.5	4.6	0.6	3.5	0.2	14	645	0.02
H. salinarum[b]	5	2.6	87	8.9	9.8	155	818	0.19
R. xylanophilus	6	3.2	79	8.2	9.6	549	290	1.9
R. radiotolerans	10	3.4	211	18	11.8	300	340	0.88
P. furiosus	3	1.9	5.3	113	0.1	14	345	0.04
T. gammatolerans	6	2.1	6.3	15	0.4	3	235	0.01

[a] Dose at which viable cells are reduced to 10% of the population
[b] From [11]

We used high-performance anion-exchange chromatography (HPAEC) to quantify compatible solutes in UFs. *R. radiotolerans* and *R. xylanophilus* UFs both contained high amounts of trehalose with 29mM and 17mM, respectively. In addition, we found mannosylglycerate (MG) in UFs of both *R. xylanophilus* (99 mM) and *R. radiotolerans* (64 mM), whereas only the *R. xylanophilus* UF contained di-*myo*-inositol phosphate (DIP) (33 mM) (Table 2). *P. furiosus* UF had 52 mM of MG and 6 mM of DIP, which was significantly more than the concentrations found in the UF of *T. gammatolerans*. Amino acid and peptide concentrations were not significantly elevated in the *Rubrobacter* species UFs or that of *P. furiosus*, when compared with *H. salinarum* UF, whereas *T. gammatolerans* UF had a significantly higher free amino acid concentration (Tables 2). Thus, the UFs of all the thermophiles reported here accumulated some small organic molecules but only the UFs of *R. radiotolerans* and *R. xylanophilus* exhibited significant amounts of Mn.

Table 2. Concentrations of amino acids, PO4, and compatible solutes in thermophiles and radiation sensitive bacteria UFs and thermophiles ethanol extracts

	Conc. In:								
	Ultrafiltrates (mM)						Ethanol extracts (µmol/mg protein)		
	Amino Acids								
Organism	Free	Total	PO_4	Trehalose	MG	DIP	Trehalose	MG	DIP
P. putida[a]	52	121	4.5	nd	nd	nd			
E. coli[a]	80	181	5.9	nd	nd	nd			
H. salinarum[a]	325	642	22	nd	nd	nd			
R. xylanophilus	87	115	10	17	99	33	1.5	3.0	0.7
R. radiotolerans	134	159	24	29	64	-[b]	1.7	2.4	nd
P. furiosus	15	35	5.4	nd	52	6	nd	0.2	0.04
T. gammatolerans	221	235	19	nd	10	2.3	nd	0.1	0.05

[a] From [11]
[b] Not detected
nd, not determined

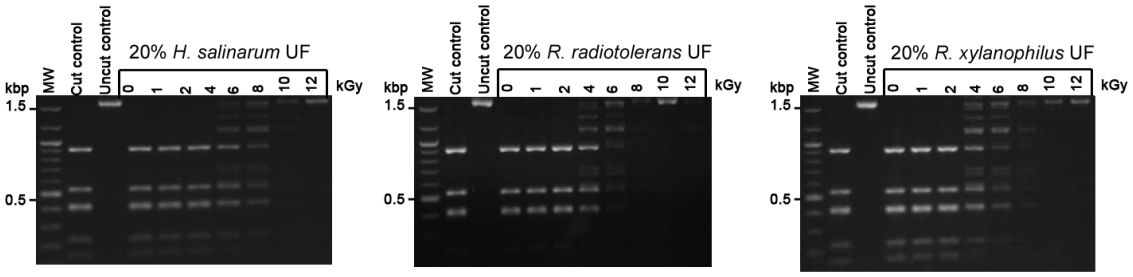


Fig. 14. Protection of enzyme activity. The restriction enzyme *Dde*I was irradiated up to 12 kGy in enzyme-free cell extracts (UFs) of *H. salinarum*, *R. radiotolerans* and *R. xylanophilus* (diluted to 0.2x). Residual restriction enzyme activity was assayed by the digestion of pUC19 plasmid DNA; fragments were analyzed by agarose gel electrophoresis. The first lanes are molecular size ladders.

To investigate the role of those small molecules in radiation resistance, we tested the ability of UFs, and of reconstituted preparations, to protect the activity of purified enzymes exposed to increasing doses of IR. *R. xylanophilus* and *R. radiotolerans* UFs provided protection of enzyme activity at doses extending to 6 and 8 kGy, respectively, which was comparable to levels of protection conferred by *H. salinarum* UF and significantly higher with the UF of IR sensitive organisms (Fig. 14). Next, we tested the compatible solutes found in the UFs and the cells of both *Rubrobacter* species for their ability to protect enzyme activity against IR, at physiologically relevant concentrations. While the phosphate buffer (P_iB) protected enzyme activity to 2 kGy, the addition of trehalose resulted in a significant increase in protection, up to 6 kGy (Fig, 15). When trehalose and P_iB were combined with 0.25 mM Mn^{2+} (determined to be physiologically relevant from the whole cell analysis) the radioprotection increased dramatically to 12

kGy. Irradiating the enzyme in P_iB and Mn^{2+} alone only protected its activity to 2 kGy and the addition of 25 mM MG or DIP did not increase protection (Fig. 15).

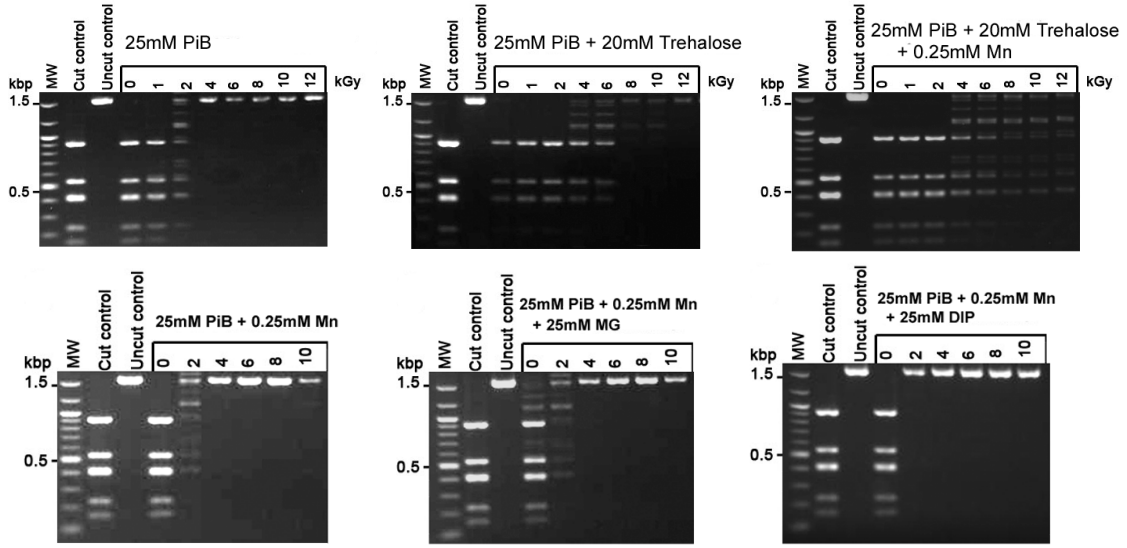


Fig. 15. Protection of enzyme activity with compatible solutes under aerobic conditions. In upper panels, the restriction enzyme *Dde*I was irradiated up to 12 kGy with 25 mM phosphate buffer (P_iB) and the addition of 20 mM trehalose and 0.25 mM Mn^{2+}. In lower panels, the enzyme was irradiated up to 10 kGy, with 25 mM PiB combined with 0.25 mM Mn^{2+}, with the addition of 25 mM MG or DIP. Residual restriction enzyme activity was assayed by the digestion of pUC19 plasmid DNA; fragments were analyzed by agarose gel electrophoresis. The first lanes are molecular size ladders.

In contrast to the *Rubrobacter* UFs, UFs of the anaerobes *P. furiosus* and *T. gammatolerans* did not protect *Dde*I activity at doses greater than 1 kGy under aerobic conditions (Fig. 16). To determine whether or not the lack of radioprotection was due to the presence of dioxygen (O_2), we tested the UFs' properties under anaerobic conditions. In the absence of O_2, UFs of *P. furiosus* and *T. gammatolerans* protected *Dde*I up to 3 kGy (Fig. 16). The addition of 0.025 mM Mn^{2+} to UFs of *P. furiosus* and *T. gammatolerans* extended protection of the *Dde*I enzyme to 5 kGy, representing an increase of 2 kGy over aerobic conditions (Fig. 16). While this Mn concentration (0.025 mM) was physiological relevant for *P. furiosus* and *T. gammatolerans*, it was 10 to 100-fold less than the Mn concentration found in the cells of the aerobic radiation resistant *Rubrobacter* species (Table 1).

We also compared the enzyme protection activity of MG and DIP in the presence and absence of O_2. Under the anaerobic conditions found in the intracellular milieu of *P. furiosus* and *T. gammatolerans*, MG protection of the *Dde*I enzyme was extended to 5 kGy, from only 1 kGy under aerobic conditions. Protection of enzyme activity was also extended under both aerobic and anaerobic conditions when the enzyme was irradiated with MG and Mn^{2+}. DIP did not show any increase in enzyme protection, either alone or in combination with MG and Mn^{2+}, but rather caused a decrease in enzyme protection. In fact, the level of protection afforded by PiB alone was identical to that with PiB and 20 mM DIP. We also found that PiB was more protective than 20 mM of MG alone. These experiments show that incubation of the enzyme under anaerobic conditions

during irradiation was the single most effective condition for extending enzyme activity to higher doses of IR.

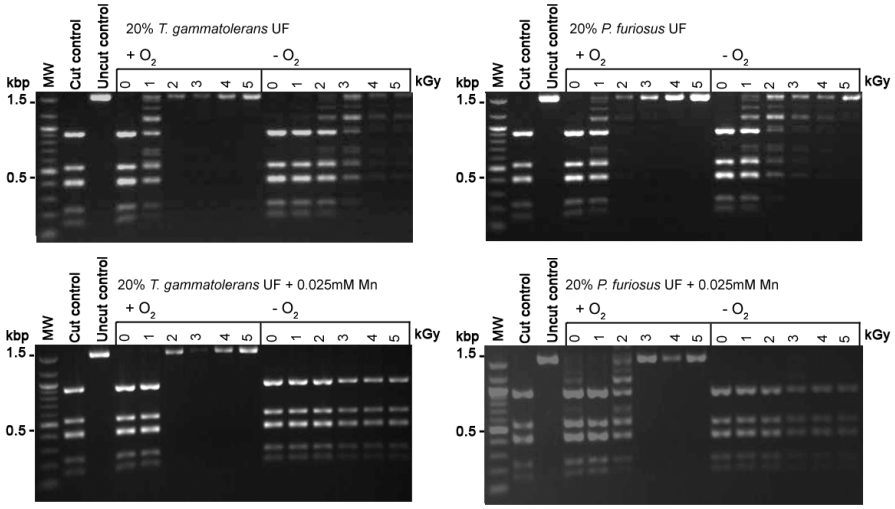


Fig. 16. Protection of enzyme activity in aerobic and anaerobic conditions. The restriction enzyme *Dde*I was irradiated up to 5 kGy in the presence or absence of oxygen in enzyme-free cell extracts (UFs) of *T. gammatolerans* and *P. furiosus* (diluted to 0.2x). Residual restriction enzyme activity was assayed by the digestion of pUC19 plasmid DNA; fragments were analyzed by agarose gel electrophoresis. The first lanes are molecular size ladders.

These experiments demonstrated that IR resistance of the thermophilic bacteria *R. xylanophilus* and *R. radiotolerans* was highly correlated to the accumulation of high intracellular concentration of trehalose in association with Mn, supporting the model of Mn^{2+}-dependent ROS scavenging in the aerobes. In contrast, the hyperthermophilic archaea *T. gammatolerans* and *P. furiosus* did not contain significant amounts of intracellular Mn and we found no significant antioxidant activity from mannosylglycerate and di-*myo*-inositol phosphate *in vitro*. We therefore propose that the low levels of IR-generated ROS under anaerobic conditions combined with highly constitutively expressed detoxification systems in these anaerobes are key to their radiation resistance and circumvent the need for the accumulation of Mn-antioxidant complexes in the cell.

1.8 Conclusions and future studies

We showed that the key processes underlying the IR resistance of *H. salinarum* are non-enzymatic and depend on the accumulation of Mn complexes, amino acids, and small peptides in the cell. Mn is a key factor for IR resistance in halophilic archaea, and in most IR resistance organisms investigated so far, therefore understanding the regulation of Mn homeostasis in extremophiles will be critical to elucidate “metabolic routes” to IR tolerance.

Radiation resistance can be manipulated by metabolic changes as demonstrated by our analysis of IR^+ isolates evolved to higher IR resistance. Our work underlined the role played by proteins involved in central metabolism and energy generation, and in the

maintenance of redox homeostasis. Increased levels of expression of proteins involved in DNA metabolism also suggested a key role for enhanced DNA repair in the IR tolerance of IR^+ mutants. Further hypothesis testing is required to characterize the metabolic pathways involved, their regulation, and to determine what mechanisms might enhanced DNA repair.

These studies underline that IR resistance is the result of the coordination of multiple, tightly regulated metabolic and DNA repair pathways that are linked to the adaptation of organisms to their environment. *Future avenues of research lie in elucidating the mechanisms that regulate Mn homeostasis in extremophiles and in determining the DNA repair mechanisms that may provide enhance tolerance to IR.*

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2. Personnel supported

PI: Jocelyne DiRuggiero, associate research professor (25% salary from AFOSR)

Ph.D. student: Kim Webb (100% salary from AFOSR)

Ph.D. student: Shuhong Lu (100% salary from AFOSR)

Research associate: Courtney Robinson (25% salary from AFOSR)

3. Publications and invited talks

Peer-reviewed publications

Webb, K., J. Wu, C.K. Robinson, N. Tomiya, Y. Lee, and J. DiRuggiero. 2013. Effects of intracellular Mn on the radiation resistance of the halophilic archaeon *Halobacterium salinarum*. Extremophiles. DOI 10.1007/s00792-013-0533-9

Webb, K.M., and J. DiRuggiero. 2012. Role of Mn^{2+} and compatible solutes in the radiation resistance of thermophilic bacteria and archaea. Archaea. 2012:Article ID 845756. doi:10.1155/2012/845756

Robinson, C.K., K.M. Webb, A. Kaur, P. Jaruga, M. Dizdaroglu, N. Baliga, A. Place, and J. DiRuggiero. 2011. A major role for non-enzymatic antioxidant processes in the radioresistance of *Halobacterium salinarum* J. Bacteriol. 193:1653-1662

Book chapters

Webb K. and J. DiRuggiero. 2013. Radiation resistance in extremophiles: fending off multiple attacks. In: Polyextremophiles Microorganisms and macroorganisms living under multiple forms of stress. Seckbach J., A. Oren, and H. Stan-Lotter (eds) Springer. pp 249-267

Kish A. and J. DiRuggiero. 2012. DNA Replication and Repair in Halophiles. In: Advances in understanding the Biology of Halophilc Bacteria and Archaea. Vreeland R.H. (ed) Springer-Verlag. pp 163-198

Invited academic talks

College of Earth, Ocean, and Environment, University of Delaware, 2013

Center for Astrobiology, University of Arizona, 2012

Space Telescope Science Institute, 2012

Institute for Genome Sciences, University of Maryland Medical School, 2012

University of Antofagasta, Chile, 2011

Harvard Origins of Life Initiative, 2011

Johns Hopkins University, Earth and Planetary Science Dept, 2011

Invited conference talks

Robinson C.K., K. Webb, and J. DiRuggiero. 2013. Molecular Basis for Protection Against Ionizing Radiation and Oxidative Stress in Halophilic Archaea. Halophiles 2013, Storrs, CT.

Robinson C.K., K. Webb K., J. Wu, and J. DiRuggiero J2012. Molecular Basis for the Ionizing Radiation Resistance of Halophilic Archaea. Extremophiles 9th International Congress. Sevilla, Spain.

Webb K., C.K. Robinson, J. Yu, and J. DiRuggiero. 2012. Molecular Basis for Protection Against Radiation and Oxidative Stress in Extremophiles. ABSciCon 2012. Atlanta, GA.

4. Collaborations

Michael Daly, Uniformed Services of Health Science: Ionizing radiation

Stuart MacNeill, St Andrew University: DNA Repair; *H. volcanii* constructs

Frank Robb, IMET, UMD School of Medicine: Protein Biochemistry

Robert Cole, Johns Hopkins School of Medicine: Proteomics

Y.C. Lee, Johns Hopkins University: Novel compounds identification